Amount of H Antigen Expressed on Circulating von Willebrand Factor Is Modified by ABO Blood Group Genotype and Is a Major Determinant of Plasma von Willebrand Factor Antigen Levels

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Abstract—To investigate whether the effect of ABO blood group on plasma von Willebrand factor (vWF) levels is mediated by the ABH antigenic determinants carried on N-linked glycans of vWF, we studied 158 group A and group O healthy volunteers. vWF antigen (vWF:Ag) and factor VIII antigen (FVIII:Ag) levels were highest in A1A1 individuals and higher in A’O1 than in A’O1 or O’O1 individuals. Plasma A transferase activity and the amount of A antigen expressed per unit vWF (AvWF) were significantly higher in A1A1 than in A’O1 individuals and higher in A’O1 than in A’O1 individuals. AvWF was correlated strongly with plasma levels of A transferase activity. Thus, we have clearly demonstrated a direct relationship between ABO genotype, A transferase expression, and the amount of A antigen expressed on circulating vWF. H antigen expression per unit vWF (HvWF) was highest in group O individuals. Among group A individuals, the pattern of HvWF expression was A’O1>A’O1>A1A1. In group O and group A’O1 individuals, HvWF was inversely correlated with plasma vWF levels. In contrast, among group A1A1 and A’O1 individuals, there was no relationship between AvWF and plasma vWF levels. These findings suggest that it is H antigen expression that mediates the ABO effect on plasma vWF concentration. (Arterioscler Thromb Vasc Biol. 2002;22:335-341.)

Key Words: ABO blood group ■ von Willebrand factor ■ factor VIII

A large adhesive glycoprotein, von Willebrand factor (vWF) circulates in the plasma as a series of heterogeneous multimers.1 It plays an important role in the interaction between platelets and the blood vessel wall and also serves as the carrier molecule for procoagulant factor VIII (FVIII).2 Both functions are essential for normal hemostasis.3 In vivo biosynthesis of vWF is limited to endothelial cells and megakaryocytes,4 but the vWF synthesized within megakaryocytes is stored within α-granules, so that the majority of plasma vWF is derived from endothelial cells.5 The normal population distribution of plasma vWF shows a wide range, with skewing toward higher levels. Deficiency of vWF (von Willebrand disease) has long been established as a cause of excessive bleeding.6 Conversely, there is accumulating evidence that elevated vWF-FVIII levels represent an important risk factor for myocardial infarction and venous thromboembolic disease.7–10 Besides the vWF gene (12p12) and the FVIII gene (Xq28), it is well established that other gene loci exert major quantitative effects on plasma levels of the vWF-FVIII complex. The most important of these loci has been shown to be the ABO blood group locus on chromosome 9q34.

The antigens of the ABO system, (A, B, and H determinants, respectively) consist of complex carbohydrate mole-
The effect of ABO blood group on plasma vWF:Ag levels may be due to altered rates of vWF synthesis or secretion by endothelial cells of a different ABO genotype. Alternatively, the effect of ABO on plasma vWF:Ag levels may be due to different rates of vWF clearance from the plasma in vivo. Thus, the lower vWF:Ag levels found in group O individuals may be the result of more rapid vWF clearance, with consequent reduction in vWF half-life. Matsui et al have demonstrated that ABH structures are carried on the N-linked oligosaccharide chains of vWF according to the blood type of the individual. Although it is not known whether the effect of ABO blood group on plasma vWF is directly mediated by these ABH structures on vWF, a recent report has shown that other carbohydrate antigenic determinants on murine vWF can significantly influence the plasma clearance rate.

ABH structures are expressed on a wide variety of human tissues, including red blood cells, platelets, epithelium, and vascular endothelial cells. However, only the circulating plasma glycoproteins expressing N-linked ABH antigens are vWF, FVIII, and α2-macroglobulin. The effect of the ABO blood group on plasma FVIII coagulant activity levels is similar to that previously described for plasma vWF:Ag levels, with significantly lower levels in group O individuals. However, no quantitative data exist comparing α2-macroglobulin levels in the different ABO blood groups.

We hypothesized that the quantitative effects of ABO blood group on plasma vWF levels are mediated by variation in the quantity of ABH antigenic determinants carried on circulating vWF. To test this hypothesis, we began by investigating the effect of ABO genotype on glycosyltransferase enzyme expression and plasma vWF levels in a series of healthy volunteers. We then quantified the amount of A and H antigenic determinants expressed per unit vWF and looked for the hypothesized correlation between these variables.

Methods

Sample Collection

One hundred fifty-eight healthy volunteers were recruited from routine blood donors at the Wessex Regional Transfusion Center, Southampton, UK. All donors were either blood group A or O and were recruited between 1970 and 1972. The study was approved by the West Research Ethics Committee, and each donor provided written informed consent. Blood was collected from the antecubital vein into Becton-Dickinson Vacutainer tubes containing 0.105 mol/L trisodium citrate. Platelet-poor plasma was obtained by centrifugation at 2000 × g for 20 minutes, with 90 minutes of collection. The plasma was then divided into aliquots and stored at −70°C.

ABO Genotyping

For each individual, the red blood cell ABO phenotype was determined by routine serological testing with the use of monoclonal anti-A and -B, respectively (Biostest AG). The ABO genotype was also established. Genomic DNA was extracted from 1 mL of citrated whole blood by using a commercial kit (Scotlab Ltd UK). ABO genotyping was performed by polymerase chain reaction amplification of exons 6 and 7 of the ABO gene, followed by diagnostic restriction enzyme digestion. Eight different primers were used to amplify 4 fragments, each spanning a different polymorphic site of the ABO gene. Primers ABO-1 and ABO-2 in conjunction with the restriction enzyme KpnI were used to differentiate A’ alleles from A, A’, B, and O’ alleles; primers ABO-3 and ABO-4 in conjunction with the restriction enzyme PvuII were used to differentiate A” alleles from A’, B, O’, and O” alleles; primers ABO-5 and ABO-6 in conjunction with the restriction enzyme AluI were used to differentiate B alleles from A’, A”, O’, and O” alleles.

vWF:Ag

Plasma vWF:Ag levels were determined by using a standard sandwich ELISA technique. Ninety-six-well plates were coated with rabbit polyclonal anti-human vWF antibodies (Dako) diluted 1:500 in 0.05 mol/L (pH 9.6) carbonate buffer overnight at room temperature. After a washing with Tris-buffered saline (TBS) containing 0.05% Tween, the plates were blocked for nonspecific binding with TBS containing 1% BSA (Sigma Chemical Co) for 1 hour. After 3 further washings, the plasma samples were added to the wells and incubated for 2 hours at room temperature. All samples were tested in duplicate at 3 different dilutions. The plates were washed with TBS/Tween and then incubated with a rabbit polyclonal anti-human vWF peroxidase conjugate (Dako) diluted 1:500 in TBS/Tween for 1 hour. After 3 further washings, the plates were incubated with a substrate solution. The reaction was stopped with 1 mol/L H2SO4, and the optical density was measured at a wavelength of 492 nm. Dilutions of 100% reference plasma (vWF:Ag 1.05 IU/mL, Immuno) were used to construct standard curves for calibration. The intra-assay and interassay coefficients of variation were <5%.

FVIII:Ag

Plasma FVIII antigen (FVIII:Ag) levels were measured by using a commercial ELISA kit (Immunozym, Technoclone) according to the manufacturer’s recommendations. All samples were measured against a reference plasma calibrated in international units per milliliter (Immunozym).

A Transferase Enzyme Expression

To measure α-1,3-N-acetylgalactosaminyltransferase (A transferase) activity, 20 μL of each plasma sample was incubated in a total volume of 100 μL, together with 5 mmol/L ATP, 20 mmol/L MnCl2, 50 mmol/L sodium cacodylate buffer (pH 7.4), 2.5 mmol/L 2-fucosylactose acceptor, and radiolabeled donor sugar in the form of 6.5 mmol/L UDP-[3H]N-acetylgalactosamine (50 000 cpm, Amer sham UK). The reaction mixtures were incubated at 37°C for 16 hours, and the radioactive product was separated on a Dowex-1 column (0.8 mL) in the formate form. The columns were eluted with 1.6 mL water, and the eluate was mixed with 4 mL of water-miscible scintillant (Pico-Fluor 40, Canberra Packard) and counted on a Beckman LS 6800 scintillation counter. Control mixtures not containing acceptor substrates were performed for each plasma sample, and any eluted counts representing breakdown of the labeled nucleotide sugar were subtracted from the product counts. Pooled A plasma (40 blood group A donors) was tested at 4 dilutions to check that the relationship between A transferase and percent UDP-[3H]N-acetylgalactosamine incorporation was linear over the assayed range.

A and H Blood Group Antigenic Determinants on vWF

A (N-acetylgalactosaminyl α-1→3)[fucosyl α-1→2] galactose) antigenic determinants on plasma vWF were measured by using a modified sandwich ELISA. ELISA plates were coated with rabbit anti-human vWF antibody (Dako), washed, and blocked with TBS containing 1% BSA as per the vWF:Ag ELISA described previously. After 3 further washings, the plasma samples were added to the wells and incubated for 2 hours at room temperature. Each plasma was tested in duplicate at 3 dilutions. The plates were washed with TBS/Tween and incubated with murine anti-A monoclonal antibody (Ortho Diagnostics), which was diluted 1:10 in TBS, for 1 hour. After 3 further washings, the plates were incubated with goat anti-mouse IgM peroxidase conjugate (Sigma), which was diluted 1:1000 in TBS, for 1 hour. After another TBS/Tween wash, peroxidase substrate solution was added and incubated in the dark for 20 minutes. The reaction was stopped with 1 mol/L H2SO4 after 3 minutes, and the optical density was measured at a wavelength of 492 nm by use of an ELISA reader. Pooled group A plasma was assayed to produce a standard curve for each ELISA.
By use of the standard curve, a value for A antigen on vWF expression was determined for each plasma sample. Plasma vWF:Ag concentration strongly influenced the amount of A antigen detected in each ELISA well ($P<0.0001$ and $P=0.74$, Spearman rank correlation). To determine the amount of A antigen expressed per unit vWF, the amount of A antigen detected was divided by the amount of vWF:Ag (see vWF:Ag ELISA) present in the ELISA well. These values of A or H per unit vWF are denoted $AvWF$ and $HvWF$, respectively. The pooled normal A plasma was assigned a value of 1 U/mL for the amount of $AvWF$.

Statistical Analysis
All statistical analyses were performed by using the SPSS statistics package (version 10.0, SPSS Inc), and statistical significance was assigned at a value of $P<0.05$. Data were not normally distributed. Consequently, nonparametric statistical analyses (Mann-Whitney and Spearman rank correlation) have been used.

Results
We investigated a total of 158 normal blood donors (82 male and 76 female). All individuals recruited into the present study were repeat donors. Previous serological testing had shown that all were either blood group A or O. The ABO genotype distribution of these individuals was as follows: 13 were $A^A$, 59 were $A^O$, 1 was $A^A$, 13 were $A^O$, and 72 were $O^O$. All donors were white, and the blood group frequencies are in keeping with this.

ABO Genotype and Plasma vWF:Ag/FVIII:Ag Levels
In keeping with previous reports, vWF:Ag levels were significantly higher in $A^A$ individuals (mean 111.8 IU/dL) than in $A^O$ individuals (mean 96.1 IU/dL, $P=0.03$) and higher in $A^O$ individuals than in $A^O$ or $O^O$ individuals (means 84.1 and 77.4 IU/dL, respectively; $P=0.03$ and $P=0.033$, Mann Whitney; Figure 1). There was no significant difference in vWF:Ag level between $A^O$ and $O^O$ individuals. Similarly, FVIII:Ag levels were highest in $A^A$ individuals (mean 105.2 IU/dL) and significantly higher in $A^O$ individuals (mean 97.9 IU/dL) than in $A^O$ or $O^O$ individuals (means 85.3 and 76.2 IU/dL, respectively; $P=0.01$ and $P=0.016$, Mann-Whitney test). Overall, there was a strong correlation between vWF:Ag and FVIII:Ag levels ($P<0.001$ and $P=0.70$, Spearman rank correlation).

Although FVIII:Ag and vWF:Ag levels were significantly different in individuals of different ABO groups, the ratio of FVIII:vWF was not significantly different between blood group A and blood group O subjects. Moreover, the FVIII:1:vWF ratios for each of the 4 genotypes studied demonstrated no significant differences ($A^A/0.95$, $A^O/1.01$, $A^O/1.01$, and $O^O/0.99$).

A Transferase Enzyme Expression
From Figure 1, it is clear that the ABO genotype exerts a dosage effect on plasma vWF:Ag and FVIII:Ag levels, such that $AO$ heterozygotes have levels intermediate between $AA$ and $O$. To determine whether the ABO genotype mediates this by exerting a dosage effect on A transferase enzyme expression, we measured plasma A transferase activities. A transferase levels were highest in $A^A$ individuals and significantly higher in $A^O$ than in $A^O$ or $O^O$ individuals ($P=0.03$ and $P=0.033$, respectively; Mann-Whitney).

Blood Group A Genotype and vWF A Antigenic Determinant Expression
To determine whether genotype at the ABO locus exerts a quantitative effect on vWF $ABH$ antigen expression, the amount of $AvWF$ was measured in the 158 plasma samples. The amount of $AvWF$ was significantly higher in $A^A$ than in $A^O$ individuals and higher in $A^O$ than in $A^O$ or $O^O$ individuals ($P=0.001$ and $P<0.001$, Mann-Whitney; Figure 3). Furthermore, $AvWF$ levels demonstrated marked variation even among individuals of the same genotype. $AvWF$ in $A^O$ individuals was low, in keeping with the low levels of A transferase activity previously demonstrated in this group.

Among $A^A$ and $A^O$ individuals, in whom accurate quantification of $AvWF$ was possible, $AvWF$ levels were strongly correlated with plasma levels of A transferase
activity \((P<0.001\) and \(\rho=0.54\), Spearman rank correlation; Figure 4). AvWF levels demonstrated the same trend as plasma vWF:Ag levels \((\lambda A'>A'O'>A'O'O'O')\). However, among \(A'A'\) and \(A'O'\) individuals, there was no correlation between \(A1\) and the plasma vWF:Ag level \((P=0.24\) and \(\rho=0.15\), Spearman rank correlation).

**ABO Genotype and vWF H Antigenic Determinant Expression**

The amount of HvWF was highest in group O individuals (Figure 5). Among blood group A individuals, the pattern of HvWF expression was \(A'O'>A'O'>A'A'\). There was a strong inverse correlation between HvWF and A transferase activity \((P<0.001\) and \(\rho=-0.59\), Spearman rank correlation) and between HvWF and AvWF levels \((P=0.0017\) and \(\rho=-0.36\), Spearman rank correlation).

Among \(A'O'\) and \(O'O'\) individuals, HvWF was inversely correlated with plasma vWF levels \((P=0.015\) and \(\rho=-0.26\), Spearman rank correlation). Moreover, within the group O individuals, HvWF also demonstrated a statistically significant inverse correlation with plasma vWF level \((P=0.021\) and \(\rho=-0.27\), Spearman rank correlation; Figure 6).

**Discussion**

The ABO blood group exerts a major quantitative effect on plasma FVIII-vWF complex levels. Evidence from twin studies has shown that 66% of the total variation is explained by the effect of the ABO blood group. Orstavik et al.\(^{13}\) have clearly demonstrated that the effect on plasma FVIII concentration is the result of a primary effect of the ABO locus on plasma vWF levels. The mechanism underlying the relationship between the ABO blood group and vWF levels remains unknown. Theoretically, the ABO blood group may influence the rate of vWF synthesis or secretion by endothelial cells or the rate of plasma clearance.

Our results are consistent with previous reports in demonstrating that both vWF:Ag and FVIII:Ag are highest in \(A'A'\) individuals and are significantly higher in \(A'O'\) than in \(A'O'O\) or \(O'O'\) individuals.\(^{17,18}\) As previously described, the normal range of vWF:Ag found in group O individuals clearly extends below 50 IU/dL (mean±2 SD, 36 to 157 IU/dL). Unless ABO group–specific vWF:Ag reference ranges are used, normal group O individuals will be diagnosed as type I von Willebrand disease.

Although the ABO blood group exerts a marked effect on plasma vWF:Ag and FVIII:Ag levels, there is no significant difference in the FVIII:vWF ratio across the ABO genotypes studied. FVIII and vWF circulate in plasma as a noncovalent 1:1 complex.\(^{30,31}\) If the effect of ABO on plasma FVIII:vWF levels is mediated by the rate of clearance of this complex, then the FVIII:vWF ratio should be the same in group O and non-O individuals. Conversely, if group O individuals have reduced endothelial cell vWF synthesis/secretion, then the ratio of FVIII:vWF in these individuals would be higher than that observed in non-O groups. Eikenboom et al.\(^{12}\) have previously established the validity of this argument by showing that the FVIII:vWF ratio is increased in heterozygous...
carriers of vWF null alleles, in whom low vWF is due to a synthetic defect. Thus, concordance in this study of the FVIII:vWF ratio in different ABO blood group phenotypes and genotypes supports the hypothesis that the effect of the ABO locus on plasma FVIII and vWF levels is mediated by altering plasma clearance rates.

Although ABH antigenic determinants have been demonstrated on the N-linked glycans of vWF, there is no previous evidence to suggest that these determinants are responsible for mediating the effect of ABO blood group on plasma vWF levels. However, it is interesting that platelet-derived vWF lacks ABH determinants and that platelet vWF concentration is not influenced by the ABO group. Our results demonstrate that the quantity of A antigenic determinant present on vWF (AvWF) is related to the ABO genotype of the individual. AvWF is highest in A' A' individuals and is significantly higher in A' O' than in A' O" individuals. Moreover, the amount of A antigen expressed on vWF demonstrates a significant correlation with the plasma level of A transferase activity. Curtis et al have recently shown that plasma levels of A and B glycosyltransferases are also correlated with the amount of A and B antigens expressed on platelets and various glycoproteins, including glycoprotein IIb. We have demonstrated that the highest levels of plasma A transferase activity are found in homozygous A' A' individuals, with significantly lower levels in A' O' individuals and barely detectable levels in A" O" individuals. This dosage effect of the ABO locus on glycosyltransferase expression levels has not been previously described, although it is well recognized for the Lewis blood group locus.

The strong correlation that we have demonstrated between plasma A transferase activity and AvWF levels could be explained by A antigen addition to vWF after secretion from endothelial cells. However, this is unlikely because the level of appropriate sugar nucleotide donors and of other cofactors in normal plasma is low. Moreover, the majority of human endothelial cells express A and B transferases appropriately, and renal vein endothelial extracts contain vWF with ABH blood group determinants. Last, vWF secreted by endothelial cells in response to 1-deamino-D-arginine vasopressin has been reported to have normal A antigenic determinant expression. Therefore, it seems likely that the explanation for the correlation is that A transferase expression levels within endothelial cells parallel the levels observed in plasma.

H antigen is converted into A antigen by the addition of N-acetylgalactosamine. Consequently, one would expect the amount of H and A antigenic determinants expressed on N-linked oligosaccharide chains to be inversely related. Indeed, our results demonstrate that the amount of HvWF is highest in O' O' individuals and lowest in A' A' homozygotes. Overall, HvWF is inversely related to plasma A transferase activity and AvWF levels. Thus, the ABO blood group locus determines A transferase expression, which subsequently determines the amount of H antigen on vWF that is converted into A.

Distinct hepatic receptors with specificity for fucose and for N-acetylgalactosamine have been characterized. Our observation that the amount of H antigen expressed on vWF is inversely correlated with vWF levels in group O individuals (in whom there is no confounding A antigen expression) and in group A' O' individuals suggests that accelerated clearance via a fucose-mediated mechanism may be the

Figure 4. Amount of AvWF against plasma A transferase activity. For each individual, plasma A transferase activity was measured by using 3H GalNAC as donor sugar, and the amount of AvWF was measured by ELISA. A' A' individuals are represented by open circles, and A' O' individuals are represented by filled circles. The amount of AvWF clearly demonstrates a strong correlation with the amount of A transferase activity present in plasma (P=0.001 and r=0.54, Spearman rank correlation).

Figure 5. Amount of HvWF against ABO genotype. The amount of HvWF was measured by ELISA. A reference pooled plasma was assayed at 4 dilutions to produce a standard curve for each ELISA. Plasma vWF in homozygous O' O' individuals had significantly more HvWF. The amount of HvWF was inversely correlated with the amount of AvWF (data not shown).
underlying explanation. Thus, in group A and B individuals, conversion of N-linked H antigen into A and B structures reduces H antigenic expression, resulting in delayed vWF clearance and higher plasma levels. Although the amount of H antigen expressed on vWF in A/O individuals is extremely low, the difference in vWF levels from other groups suggests that it is still significant. The correlation coefficient for the relationship between HvWF and vWF:Ag level is relatively low, indicating that only a small proportion of the total variation in vWF:Ag levels in these individuals can be attributed to the amount of H antigen expression. This finding is consistent with the estimation of Orstavik et al,13 who reported that only 30% of the total genetic variation in plasma vWF suggests that other mechanisms of clearance exist, although the lack of correlation between AvWF and plasma vWF concentration indicates that it is unlikely to be via an N-acetylgalactosamine receptor.

A marked variation in the amount of A antigen expressed on the surface of platelets of normal group A individuals has recently been reported.34 Our data confirm that there is also a large variation in the amount of A antigen on plasma vWF and indicate that this variation has at least 2 origins. First, we have shown that the amount of H antigen expressed on group O vWF can vary over a wide range. Because it appears that most H structures are converted into A structures in A/A’ and A/O’ individuals, a variation in the amount of H availability is likely to be a major determinant of A antigen expression. The origin of the variation in H antigen expression may lie among the many glycosyltransferases and glycosidases required for synthesis of complex N-linked glycan structures.33 Second, we have shown that A transferase enzyme levels can vary significantly even within group A genotypes. The regulatory elements controlling A transferase expression have not been fully characterized. Moreover, the wide variation that we have observed in plasma A transferase activity levels could also result from differences in the rates of cellular secretion or plasma clearance of the transferase enzyme.

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